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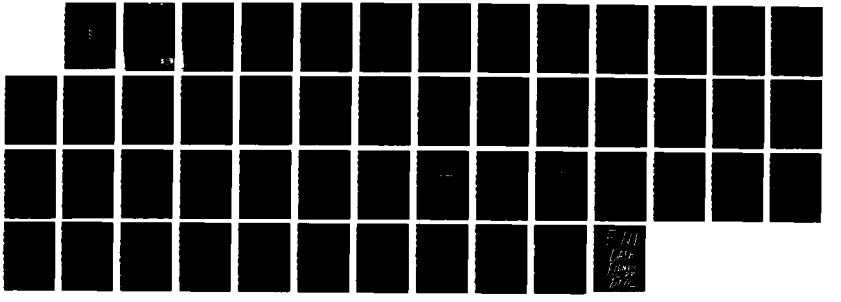

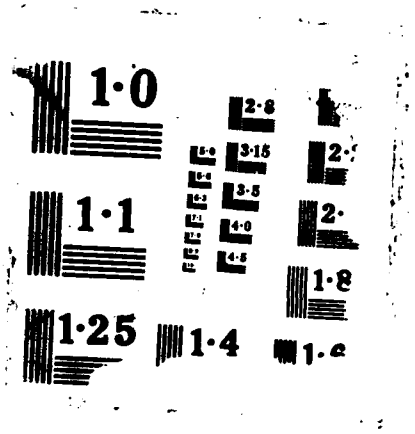
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# AUTOREGULATION OF NEUROMUSCULAR TRANSMISSION BY NERVE TERMINALS



ANNUAL REPORT

GEORGE G. BIERKAMPER

SEPTEMBER 1, 1985

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findings with equimolar concentrations of nicotine and carbamylcholine. The natural agonist, ACh, provoked frequency-dependent negative feedback on subsequent release when the neuromuscular junction concentrations of ACh were raised with neostigmine or other acetylcholinesterase (AChE) inhibitors. The nicotinic antagonists d-tubocurarine (dTC; $10^{-7}$M and $10^{-8}$M) and alpha-bungarotoxin (aBGT; 50ug) induced an increase in ACh release from the VPRH during stimulation. dTC appeared to antagonize negative feedback at low doses ($10^{-8}$M), as evidenced by a partial restoration of FC during agonist-induced negative feedback. FC and intracellular recordings from the VPRH demonstrated that the frequency-dependence of partial dTC neuromuscular blockade is associated with a prejunctional mechanism, i.e., a prejunctional cholinoceptor. Backfiring (antidromic action potentials) in the mouse phrenic nerve as a result of AChE inhibition has been linked to a prejunctional mechanism requiring the release of ACh. dTC antagonized stimulus-induced backfiring in the presence of neostigmine implicating ACh feedback on the nerve terminal. Rapid vascular perfusion of a concentrated bolus of ACh induced transient backfiring in the mouse phrenic nerve. Prevention of ACh release by botulinum toxin or tetanus toxin eliminated backfiring. Further experimentation on prejunctional mechanisms focused on the role of calmodulin and the calcium antagonist verapamil in local modulatory mechanisms of neuromuscular transmission. Subchronic exposure of the rat to DFP resulted in residual alterations in neuromuscular transmission in the isolated VPRH. Negative feedback was present as determined by FC without additional AChE inhibitor in vitro and was antagonized by dTC. A sensitive chemiluminescent method for picomolar amounts of ACh has been developed and implemented in this laboratory for routine measurement of ACh in pefusate from VPRH. In conclusion, the results of this investigation support the hypothesis that ACh release at the motor nerve terminal is modulated by local mechanisms capable of acting independently or interdependently of CNS input. These mechanisms appear to be sensitive to the immediate environment of the nerve terminal. One mechanism which appears to be important in the regulation of ACh release is a prejunctional nicotinic cholinoceptor on nerve terminals, acting in a negative feedback mode. Stimulation of the receptor with nicotinic agonists decreases stimulated ACh release; antagonism of the receptor with nicotinic antagonists increases ACh release. Negative feedback by excess poststimulus ACh in the junction is dose- and frequency-dependent; its modulatory role is substantial enough to affect muscular contraction in the VPRH. Results with verapamil and calmodulin inhibitors indicate that calcium plays an interdependent role with the prejunctional cholinoceptor in the regulation of ACh release. Prejunctional cholinoceptor activity appears to be separate from precursor control, i.e., choline availability. Acute and chronic AChE inhibition significantly affects negative feedback through the prejunctional cholinoceptor and alters ACh release. Therefore, the cumulative results from the project support the hypothesis that the nerve terminal possesses local mechanism for modulating ACh release in response to changing physiological or pharmacological demands and in the presence of AChE inhibitors.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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BODY OF REPORT

## I. Statement of Objectives

This project examines the hypothesis that motor nerve terminals have the capacity to modulate neuromuscular transmission independently of and/or interdependently with central nervous system (CNS) regulation. That motor nerve terminals "fine tune" junctional transmission is a new concept which diverges from the traditionally held view that an equal and fixed amount of transmitter is released into the neuromuscular junction by each nerve impulse. The concept of a variable gating mechanism for modulating acetylcholine (ACh) output from motor nerve terminals, and thereby muscular response, introduces the exciting possibility that specific pharmacologic agents can be designed to correct functional disorders of neuromuscular transmission that result from certain human disease states or from intoxication by chemical agents and drugs.

The objective of this study is to investigate three mechanisms through which ACh release may be modulated prejunctionally: (1) prejunctional cholinoceptor regulation of ACh, (2) modulation of ACh release through preconditioning patterns of nerve stimulation, and (3) precursor-induced alterations of ACh release. The relative importance and interrelationships of these mechanisms in comparison to other local or distal regulatory mechanisms of ACh output at the neuromuscular junction are contemplated in the process of this experimentation.

## II. Introduction and Background

This report concerns the final year of research effort in the contract. Experimentation has been conducted in all areas of the specific aims of the original proposal with considerable emphasis on anticholinesterase-induced backfiring in the phrenic motor nerve, prejunctional pharmacology, and exploration of calmodulin-mediated control of ACh release.

The primary mammalian model system of neuromuscular transmission used throughout this investigation has been the vascular perfused rat phrenic nerve-hemidiaphragm preparation (VPRH). In addition, the mouse hemidiaphragm has been used in the experiments related to antidromic activity in the phrenic nerve due to ease of use and low expense. The $^{125}$I-alpha-bungarotoxin (aBGT) binding studies have been performed in rat sciatic nerve because of the anatomy and ease of ligation in situ. ACh release has been measured directly by radioenzymatic and chemiluminescence assay and indirectly by either intracellular recording or force of contraction (FC) measurements. The new chemiluminescence method of ACh measurement has recently replaced the routine radioenzymatic assay and the high performance liquid chromatography (HPLC) method investigated earlier in this project.

## III. Results

### A. Agonist and antagonist interactions with the putative prejunctional cholinoceptor

Our hypothesis states that nicotinic agonists in sufficient concentrations will activate a prejunctional cholinoceptor on the motor nerve

terminals and under higher frequencies of stimulation will cause a decrease in the amount of ACh released per impulse. Conversely, a block of the prejunctional cholinoceptor by a nicotinic antagonist will prevent negative feedback and result in an increase in the amount of ACh released per impulse. FC measurements, direct assay of ACh release from the VPRH, and intracellular recording from the neuromuscular junction have been used to assess the effects of nicotinic agonists and antagonists in modulating ACh release through the putative prejunctional cholinoceptor. Nicotine, carbachol, ACh and suberyldicholine have been used as agonists.[1,2] Neostigmine (NEO) and related acetylcholinesterase (AChE) inhibitors have been used to raise pharmacologically the poststimulus levels of ACh (the natural agonist) at the neuromuscular junction.[2] Curare, aBGT, hexamethonium, and edrophonium have been used as potential nicotinic antagonists.[2]

**1. Nicotinic Agonists**

Suberyldicholine (SDCh; 10 uM), a potent nicotinic agonist, caused a 39% decrease in stimulated ACh release (Fig. 1) as measured by radioenzymatic assay. This effect was readily reversible on washout of the drug from the preparation. This depression of ACh release is even more pronounced than the action of equimolar concentrations of nicotine (Fig. 2)[2]. The action of SDCh corroborates, therefore, the previous studies with carbachol[2] and nicotine[2]. The ability of all three compounds to reduce stimulated ACh release lends support to the theory that a prejunctional negative cholinoceptive modulator is operational at motor nerve terminals.

A recent series of experiments with nicotine using a modified ACh release/assay protocol reveals a consistent trend in non-quantal ACh release which warrants caution in the above interpretation. Figure 3 illustrates the overall depression in ACh release when 10 uM nicotine is present in the VPRH. Stimulation (10Hz) occurred in only the third 15 min collection period. A similar 30% reduction in ACh release was observed in stimulated release as compared to Fig. 2. However, "resting" release revealed a consistent, though statistically insignificant, decrease. We have shown earlier[3] that resting release is composed of non-quantal (>98%) and quantal (mepps; <2%) ACh release. It would be very curious, indeed, if the nicotinic agonists reduced non-quantal release, since the role of non-quantal release has yet to be determined. Our experience with the VPRH preparation suggests that such consistent trends in ACh release in one direction, even though insignificant at the 0.5 level compels further experimentation. Based on our total information to date it appears unlikely that changes in resting release explain the negative feedback action of nicotinic agonists. However, modifications in resting release may be an underlying secondary consequence of nicotinic stimulation which is worth pursuing in future experiments.

In conclusion, the nicotinic agonists, nicotine and suberyldicholine, reduce the concentration of ACh in perfusate from the VPRH during and immediately following nerve stimulation. The depression in stimulated release is readily reversible by washout of the drug. These results confirm our earlier findings [1,2]. Nicotine ($10^{-5}$M) was observed to induce a trend towards a lower amount of ACh release when the protocol was altered to one release period during an experiment and when measurement of ACh was accomplished by the new chemiluminescence assay. The change in release protocol revealed more information about resting release and suggests that nicotine may also affect

non-quantal ACh release in addition to its apparent action of evoked quantal release.

### 2. Nicotinic Antagonists

The nicotinic antagonists, d-tubocurarine (dTC) and alpha-bungarotoxin, were shown to enhance stimulated ACh release from the VPRH in our previous report[2] as predicted by our working hypothesis. Work with antagonists this year has primarily focused on low concentrations of dTC ($<10^{-7}$M) as an antagonist to negative feedback by nicotinic agonists. Preliminary assays indicate that 0.1 uM dTC may antagonize the depressive effect of nicotine (10 uM) on stimulated ACh release from the VPRH. Confirmation of this observation will require further experimentation. A positive result, however, would corroborate the antagonistic action of dTC on agonist-induced depression of FC at 10 and 30 Hz as demonstrated previously[2]. In addition, dTC has been studied by intracellular recording techniques in association with the frequency-dependence of negative feedback. These data are discussed in section D below. Finally, dTC has been employed as a nicotinic antagonist to block AChE-induced backfiring in the phrenic motor nerve (Section F). Its use has strengthened the theory that backfiring is mediated through prejunctional nicotinic cholinoceptors.

Finally, the antagonists dTC ($10^{-6}$M) and aBGT increase stimulus-evoked ACh release from the VPRH as measured directly by radioenzymatic assay[2]. Moreover, low doses of dTC ($10^{-7}$-$10^{-8}$M) antagonize the negative effects of cholinergic agonists on the FC in stimulated VPRH. It is unclear whether increases in ACh release (20-25%) strengthen muscular FC because of the so-called "margin of safety." However, select prejunctional cholinoceptor antagonism may have the potential of raising ACh release in conditions of compromised release. dTC (0.1 uM) may antagonize the depression in stimulated ACh induced by nicotine (10 uM) as measured in the VPRH by chemiluminescence assay. These preliminary results strengthen the theory that ACh release may be modified prejunctionally by pharmacological agents and/or during physiological conditions which create an imbalance in the amount of stimulated ACh release (natural venom or toxin exposure; certain disease states) or hydrolysis (e.g., AChE inhibition).

## B. The neuronal role of calmodulin in neuromuscular transmission.

Depolarization evoked ACh release from phrenic motor nerves requires calcium[3]. Calcium enters the nerve terminal at the time of depolarization of the terminal branches by the action potential. The amount of transmitter release appears to be dependent on the concentration of $Ca^{++}$ entering the cell. If one were to design a mechanism to modulate ACh release, regulation of calcium entry would be a logical control point. Thus, we have begun to explore the possibility that the prejunctional receptor could be associated with a voltage-dependent calcium channel. This is being accomplished, in part, in the backfiring studies in mouse hemidiaphragm-phrenic nerve (Section F). In addition, this last year's effort has included more experimentation to learn the fate of $Ca^{++}$ once it enters the terminal in response to depolarization. The intraterminal mechanism by which $Ca^{++}$ influx effectuates transmitter release is not understood. Recent studies reveal that the $Ca^{++}$ binding protein, calmodulin, may play a role in the excitation-secretion

coupling mechanism[4,5,6]. In theory, $Ca^{++}$-calmodulin regulates ACh release by altering cAMP concentrations[7] in the nerve terminal via activation of a $Ca^{++}$ dependent adenylate cyclase[8]. A transient rise in cAMP levels would activate a protein kinase that mediates phosphorylation of a protein associated with the release mechanism[5,9] (Fig. 3). Inhibition of calmodulin would be expected to decrease this $Ca^{++}$-dependent increase in cAMP and thus, to depress ACh output. In contrast, elevating cAMP levels by the activation of adenylate cyclase, the inhibition of phosphodiesterase or the introduction of dibutyryl-cAMP (db-cAMP) would be expected to antagonize the effects of calmodulin inhibition. In the present study, these theories were partially tested in an isolated neuromuscular model system using four putative calmodulin inhibitors and three cAMP "enhancers," individually or in combination.

The VPRH preparation from Long Evans hooded male rats (275-350 g) was used in these experiments. FC was measured via a force transducer during indirect stimulation of the nerve (1.2V; rectangular pulses; 0.2 ms duration) or by transmural stimulation of the muscle directly (100V; supramaximal) as described previously[10]. In 24 additional experiments, the release of ACh was measured in perfusate from the VPRH by chemiluminescent assay (described in Section H below). Recovery of released ACh required the presence of the acetylcholinesterase inhibitor, NEO ($10^{-5}$M). These preparations were continuously stimulated at 10 Hz (indirect) for 75 min to establish a steady state control level of ACh release; then drug was introduced for 45 min. Untreated control VPRH's maintained a constant release of $6.52 \pm 0.55$ pmol/min/hemidiaphragm from 60-180 min.

The neuroleptic phenothiazines, fluphenazine (FLU), chlorpromazine (CPZ) and trifluoperazine (TFP), with known anti-calmodulin activity[11] induced a decrease in FC via indirect, but not direct muscle stimulation at $10^{-4}$M (Table 1). These compounds had no effect on FC at $10^{-6}$M and had only marginal, statistically insignicant depressive effects on FC at $10^{-5}$M. N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide hydrochloride (W-7), a specific calmodulin antagonist[12], produced a trend towards depressing both indirectly and directly evoked FC (Table 1). Depression of FC by W-7 by direct stimulation was not unexpected since it inhibits calmodulin stimulated myosin light chain phosphorylation [13]. The limited availability of the drug prevented additional experiments to reduce inter-experiment variability noted in the first 3 trials. Washout of these 4 anti-calmodulin compounds for 30-45 min resulting in only a marginal reversibility of less than 10% increase in FC.

Three compounds which elevate intracellular cAMP levels were tested separately to determine their effects on normal FC at 0.5Hz. Forskolin ($10^{-6}$M,

n=2; $10^{-5}$M, n=2; $10^{-4}$M, n=1), an adenylate cyclase activator, had no effect on FC. db-cAMP ($10^{-5}$M, n=2; $10^{-4}$M, n=1) increased FC by 19% and 44%, respectively, during indirect stimulation; direct stimulation provoked similar increases. Theophylline (THEO; $10^{-4}$M, n=2; $10^{-5}$M, n=2), a phosphodiesterase inhibitor, increased FC by 23% and 42%, respectively, for indirect, and similarly for direct, stimulation.

THEO ($10^{-3}$M) kept FC at control levels in the presence of all 4 calmodulin inhibitors ($10^{-4}$M; Table 1). db-cAMP ($10^{-4}$M) did not significantly antagonize the decreases in FC induced by FLU ($10^{-4}$M) and CPZ ($10^{-4}$M; however,

1 mM db-cAMP antagonized the depressive actions of FLU ($10^{-4}$M) and CPZ ($10^{-4}$M). Forskolin ($10^{-5}$M) completely antagonized the depressive effects of FLU ($10^{-4}$M) on FC.

The stimulated release of endogenous ACh from the VPRH was significantly decreased by FLU ($10^{-4}$M) and CPZ ($10^{-4}$M) (Table 2; no other concentrations or drugs were tested due to the lengthy procedure of the assay). THEO ($10^{-4}$M) antagonized the depressive effect of FLU on stimulated ACh release. Washout of either FLU or CPZ for 30 min failed to reverse the depression. These release experiments were a critical test to prove that the FC data was truly reflective of alterations in ACh release. More ACh release studies are planned in future projects.

These results demonstrate that the calmodulin inhibitors FLU, CPZ, and TFP depress neuromuscular transmission by a prejunctional mechanism. W-7 appears to reduce FC by both a prejunctional and a postjunctional mechanism. In support of our hypothesis, the prejunctional mechanism appears to involve an inhibition of neurotransmitter release, since we have observed a decrease in ACh release in the presence of FLU and CPZ. In addition, we have demonstrated that drugs which increase cAMP levels within the nerve terminal antagonize the calmodulin inhibitors in equimolar or greater concentrations. These results lend support to the theory that stimulus-evoked ACh release is regulated by a cascading pathway involving $Ca^{++}$-calmodulin-adenylate cycle-cAMP-protein kinase-protein phosphorylation-ACh release mechanism as diagrammatically represented in Fig. 4. These data must, however, be viewed cautiously for two reasons: 1) calmodulin and cAMP may not be directly coupled in the excitation-secretion mechanism for ACh release; thus, we could be indirectly compensating for calmodulin inhibition via another independent modulatory mechanism, and 2) the concentrations of drugs required to induce changes are quite high. Other investigators in the calmodulin area view these concentrations as acceptable in order to get the drug to the intraterminal site of action. Nonetheless, one must consider the possible inhibition of other neuronal enzymes at these high doses[14], or the membrane stabilization or channel blocking effects of the phenothiazines which would impede cation flux across the depolarized membrane[14,15]. Furthermore, it remains to be shown that this calmodulin system is directly coupled to the depolarization induced entry of $Ca^{++}$ or to the prejunctional nicotinic receptor on motor nerve terminals.

### C. $^{125}$I-aBGT binding sites in sciatic nerve.

$^{125}$I-aBGT binding sites were shown to be transported in an orthograde and retrograde manner in ligated rat sciatic nerve in a previous annual report[1]. Graphs of the accumulation of the binding over time and the displacement of binding by cholinergic drugs were presented in this report.[1] Further study confirmed the nicotinic nature of the binding sites and revealed binding characteristics more similar to the CNS nicotinc receptor than the skeletal muscle receptor.[2] Axonally transported toxin binding sites may correspond to those previously localized to the plasma membrane of peripheral nerve axons and on the terminals of motor neurons. Thus, these results lend support to the hypothesis that nerve terminals contain nicotinic cholinoceptors.

These studies have been extended beyond the original aim which was to provide evidence that the putative prejunctional nicotinic receptors are

axonally transported. We have also explored the possibility of using this "receptor" binding method in studying axonal transport in the cholinergic system during toxicological insult.

In brief, our quantitative autoradiographic technique has been utilized to evaluate the effects of various toxic agents on the axonal transport of alpha-bungarotoxin binding sites in rat sciatic nerve. The agents used have been shown elsewhere to produce peripheral neuropathies. Treatment of rats with B,B'-iminodipropionitrile (IDPN) produced a marked reduction in the amount of binding sites, reflected as silver grain densities, accumulating at both sides of a ligature placed around the sciatic nerve for eight hours in vivo (Table 3). A similar finding was observed when the nerve was crushed seven days prior to ligation. In contrast, acrylamide treatment produced a reduction of binding sites accumulating distally to the ligature only (Table 3). No changes were seen after p-bromophenylacetylurea (BPAU) treatment (Table 3). We conclude that this technique is valuable in the study of experimental neuropathies induced by chemical agents as a method to quantify the axonal transport of specific proteins.

**D. Frequency-dependence of negative feedback and ACh release: the action of curare**

In a physiologically active neuromuscular preparation which is required to release a significant amount of ACh in response to 10-40 Hz of continuous nerve stimulation, negative feedback of ACh would be predicted. At higher frequencies of stimulation, endplate potential (EPP) amplitude would be expected to decrease as less ACh was released. d-Tubocurarine (dTC) would be expected to have a dose-dependent action on the amount of ACh released by antagonizing the putative prejunctional cholinoceptor. Higher concentrations of dTC should antagonize the negative feedback and flatten the amplitude vs. frequency response curve.

The frequency dependence of neuromuscular block in the presence of dTC was studied in the VPRH preparation by intracellular recording. Preparations were stimulated continuously via the phrenic nerve (supramaximal; ~5 V, 0.2 msec; via optical isolation) at either 10 or 20 Hz in the presence of curare. Within one minute, contraction ceased and conventional intracellular recording of EPPs was accomplished using glass microelectrodes filled with 3M KCl (5-15 Mohm). Since impalement of muscle cells could not be maintained with intermittent stimulation, preparations were stimulated continuously at either 10 or 20 Hz (holding frequencies) with intermittent stepping to test frequencies (2.5, 5, 10, 20 and 40Hz) for two minutes. For an experiment at a given holding frequency, mean EPP amplitudes were calculated from a single cell every 3 minutes. Means were calculated from EPPs which were sampled over a 90 second period with the aid of the Smartscope Waveform Analyzer (T.G. Brandon Corp., Portland, OR). Mean EPP amplitude at a test frequency was determined 30 seconds after the start of that frequency. The mean EPP amplitude during a period at the holding frequency was always determined before the mean at a test frequency. For each test frequency, the mean EPP amplitude relative to the mean EPP amplitude of the immediately preceding hold period was calculated (mean EPP test/mean EPP hold). Data were analyzed by linear regression by least-squares.

In the presence of dTC ($5 \times 10^{-7}$M) an increase from the holding frequency

decreased EPP amplitude while a decrease in frequency increased EPP amplitude. This relationship between neuromuscular block and stimulation occurred at both holding frequencies (10 and 20 Hz; Fig. 5). It was also observed that stimulation can be maintained at the holding frequencies for 20 minutes or more with little change in EPP amplitude, as shown in the representative experiments presented in Fig 5.

Relative EPP amplitudes decreased linearly with the log of stimulation frequency regardless of whether the holding frequency was 10 or 20 Hz (Fig. 6). Further, the slopes of the least-squares regressions of relative EPP amplitude vs. log of frequency were not significantly affected by the holding frequency (-168 when the holding frequency was 20 Hz; -153 at 10 Hz). This log-linear relationship between stimulation frequency and neuromuscular block extended from sub-tetanic (5 Hz) to tetanic frequencies of stimulation (40 Hz). When the stimulation frequency was less than 1 Hz, muscle movement made cell impalement impractical at this concentration of dTC ($5 \times 10^{-7}$M).

An additional series of experiments tested the hypothesis that different concentrations of dTC would alter the frequency-dependence of neuromuscular block. At both a lower concentration ($2.5 \times 10^{-7}$M, 20 Hz holding frequency) and a higher concentration ($1.5 \times 10^{-6}$M, 10 Hz holding frequency) of dTC, EPP amplitude was less at higher frequencies (Fig. 7). The slopes of the least-squares regression line of relative EPP amplitude to log of stimulation frequency were significantly different, the slope being less steep at the higher concentration (slopes: -201 at $2.5 \times 10^{-7}$M; -94 at $1.5 \times 10^{-6}$M; Fig. 8). Since the holding frequency does not affect the slope of the regression line, we conclude that increasing concentrations of dTC decrease the frequency-dependence of neuromuscular block. This is an important observation (i.e., the flattening of the Epp amplitude vs. frequency response curve) inasmuch as supports the hypothesis that dTC has a prejunctional action.

Thus, endplate potential amplitude decreased as the frequency of stimulation increased (2.5 to 40 Hz). This dependence decreased as dTC concentrations increased over the range of $2.5 \times 10^{-7}$M to $1.5 \times 10^{-6}$M. Hence, dTC most likely interferes with the theoretical negative feedback mechanism (e.g., prejunctional cholinoceptors) under study. The demonstration of a frequency-dependent dTC block at tetanic frequencies is also consistent with the channel-blocking hypothesis of tetanic fade [16] but, it is evident that this hypothesis does not provide a complete explanation of frequency-dependence of neuromuscular block in the presence of dTC. Indeed, several experimental observations are not consistent with expected consequences[17] of dTC block of postjunctional ACh channels: 1) the frequency-dependence of neuromuscular block is not enhanced by increased dTC concentration (present results); 2) low doses of cholinesterase inhibitors do not increase tetanic fade[18]; 3) tetanic fade does not depend upon resting membrane potential[19]; and 4) endplate currents produced by ACh ionophoresed at tetanic frequencies in the presence of dTC do not exhibit fade.[20] Thus, from both the data from this report and that of others, it is evident that the postjunctional channel-blocking hypothesis does not sufficiently explain frequency-dependent neuromuscular block in the presence of dTC. It is concluded that dTC may influence neuromuscular block via a prejunctional action, affecting ACh release. This conclusion is in support of the central theme of this project, i.e., ACh release is modulated via a prejunctional nicotinic cholinoceptor.

**E. Effect of organophosphate agents on choline efflux from VPRH and its influence on ACh release**

ACh synthesis by motor nerve terminals requires an adequate supply of its precusor, choline. We have found that diisopropylfluorophosphate (DFP), an AChE inhibitor commonly used in ACh release studies, reduces the rate of endogenous choline efflux from the VPRH. This was determined in the VPRH as setup to measure ACh release except that instead the concentration of free choline in the perfusate was measured. The HPLC method originally developed (see previous annual report[1]) for the measurement of ACh was adapted for the routine measurement of choline. Perfusion of the isolated hemidiaphragm with 10 uM or 100 uM DFP reduced choline efflux by 39% and 69%, respectively. DFP administration to rats (6mg/kg) also lowered the in vitro release of choline by 33%. Triortho-cresyl phosphate (TOCP; 10 uM), a neurotoxic organophosphate agent structurally related to DFP, also induced a significant decrease in choline efflux from the VPRH. However, paraoxon (10 uM) did not alter the efflux of choline from the VPRH. The rate of ACh release from hemidiaphragm preparations perfused with DFP was significantly lower than the rate of release from preparations perfused with physostigmine, an AChE inhibitor which had no effect on choline efflux. The addition of choline (10-30 uM) to the perfusion medium restored the rate of ACh release from DFP-treated hemidiaphragms but did not further elevate ACh release from physostigmine-treated preparations. These results demonstrate that DFP inhibits choline efflux from the isolated hemidiaphragm and futher suggest that, by limiting the availability of choline for ACh synthesis, DFP reduces the rate of ACh release in vitro. We speculate that DFP and TOCP may inhibit the metabolism of phospholipids containing choline, e.g., phosphatidylcholine.[21] It would be prudent to investigate the observation that DFP and TOCP, but not physostigmine or paraoxon, interfere with choline efflux in terms of the delayed peripheral neuropathies caused by the former, but not the latter. Perhaps the neuropathy involves phospholipid metabolism. This choline efflux work has recently been published.[22]

**F. Antidromic activity (backfiring) in phrenic nerve in the presence of an anticholinesterase agent**

The motor nerve normally conducts action potentials in an orthodromic direction, leading to transmitter release from the nerve terminal and activation of the muscle fibre. Following cholinesterase inhibition, however, antidromic action potentials (backfiring) can be recorded in the motor nerve. This activity arises both spontaneously and in response to a normal orthodromic volley elicited by electrical nerve stimulation (stimulus-induced antidromic activity or SADA).

Backfiring was first reported in the early 1940s[23,24] but the mechanism of its origin is still not understood. It has been suggested that cholinoceptive sites exist on the motoneuron terminal and that these sites are responsible for backfiring as well as other physiological phenomena.[18,25,26,27,28,29] Alternatively, Katz[30] suggested that antidromic action potentials may be generated by an increased potassium efflux from the muscle during cholinesterase inhibition. Before these hypotheses can be properly addressed, it is first necessary to determine whether antidromic activity is due to acetylcholine (ACh, after cholinesterase inhibition)[23,24,31,32,33]; or due to a direct action of the anticholinesterase

agent on the nerve terminal, as suggested by some authors[26,28,34,35,36,37,38]. We have employed botulinum toxin (BOT), an agent that specifically and irreversibly blocks ACh release from the nerve terminal by interfering with quantal release mechanisms[39,40,41,42], to explore this question. We have tested whether antidromic activity after neostigmine (NEO) treatment could be elicited in neuromuscular preparations in which cholinergic transmission was virtually blocked with BOT.

The mouse phrenic nerve-diaphragm preparation has been used in all of the backfiring studies due to moderate expense as compared to rat costs and ease of use of the preparation. Female Swiss mice (20-40 g) were anesthetized with chloral hydrate (0.4 mg/g) and the left phrenic nerve-hemidiaphragm was quickly removed and placed in a HEPES-buffered physiological solution (composition in mM: n-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid 6.0, NaCl 114.0, KCl 3.5, $CaCl_2$ 2.5, $MgCl_2$ 1.0, glucose 11.0; pH 7.4) which was continuously bubbled with 100% $O_2$ at room temperature ($20\pm2^{o}C$). The hemidiaphragm was then cannulated via the central diaphragmatic vein with polyethylene tubing (PE-10) tipped with a 30 gauge stainless steel tube and perfused with oxygen-supersaturated physiological solution at a continous rate of approximately 0.05 ml/min[43]. All experiments were performed at room temperature (20-22°C). The cannula was secured in place with surgical silk and the diaphragm was pinned to a layer of Sylastic in a polystyrene chamber. To isolate the electrical activity of the nerve, a small amount of paraffin oil was placed above the solution which bathed the preparation. Once the diaphragm was cannulated and the electrodes were in place, the preparation was allowed to stabilize for 5-10 min before the start of the experimental procedure.

The physiological solution was driven through the vasculature of the diaphragm by an innovative constant pressure perfusion system designed to provide oxygen to the preparation without the necessity of bubbling the bath. This method also allows for the rapid infusion and wash-out of drugs. Test solutions were introduced via the cannula from one of four alternative reservoirs under the same oxygen pressure. The reservoirs were connected to the cannulae via a 4-way micro-stopcock (HV D4-F, Hamilton, U.S.A.). The distance between the stopcock and the preparation was minimized (10-15 cm) to reduce dead space.

The phrenic nerve was stimulated by means of a pair of wire electrodes (2-3 mm apart) with a square-wave pulse (0.01-0.1 msec duration, supramaximal voltage) delivered by a stimulator (S-48, Grass, U.S.A.). Nerve activity was recorded with a pair of silver wire electrodes (2-3 mm apart) placed distally to the stimulating site, amplified (A.C.amplifier; Model P-16, Grass, U.S.A.), and visualized on a storage oscilloscope (D-11, Tektronix, U.S.A.). Permanent traces were obtained on Polaroid film using a Tektronix C-54 camera or Smartscope transient recorder. The stimulating and recording electrodes were immersed in paraffin oil which overlaid the preparation. This method was effective in minimizing the stimulus artefact.

Conventional intracellular glass microelectrodes (10-25 MOhms) containing 3 M KCl were used to record muscle end-plate activity from the diaphragm. The end-plate potentials recorded by the microelectrode were led to a unity gain amplifier (S-7000, World Precision Instruments, U.S.A.) and displayed and recorded on the oscilloscope.

Initial control experiments established a protocol of a 10 min NEO (2uM) application through the perfusate followed by a 30 min wash period with normal physiological solution. This method results in reasonably constant levels of backfiring for the entire 40 min experimental period during which the action of blocking agents could be reliably tested.

Botulinum toxin treatment was used to block ACh release. Mice were treated with a single intraperitoneal injection of 100 ug ($10^5$ $LD_{50}$) of BOT 40 min prior to dissection. This treatment has been shown to produce a highly effective blockade of quantal acetylcholine release[44]. Diaphragms from these animals were rinsed thoroughly in physiological solution prior to cannulation.

The results of these experiments are divided into 4 subsections and are discussed below:

Production of backfiring. Stimulus-induced antidromic activity could be elicited in all the phrenic nerves of 47 control preparations tested within one minute after initial infusion of NEO (2 uM) (Fig. 9). This activity usually consisted of single and compound action potentials (30-200 uV) lasting for up to 60 milliseconds following the conditioning volley (Figs. 9 and 10). The levels of backfiring varied from animal to animal but usually remained constant for each preparation for the 40 minute experimental period (10 min NEO, 30 min wash). Evoked backfiring was tested every 2 minutes by stimulating the nerve 3 times, 10 seconds apart (Fig. 10). This protocol was adopted to observe the possible effects of drug treatments on a characteristic feature of SADA at room temperature: at stimulation rates below 0.1 Hertz the number of antidromic spikes remains constant with succesive stimuli[45].

In addition to SADA, spontaneous antidromic action potentials of low and variable frequency were detected in all the control preparations following NEO administration (Fig. 11). These spikes were of small amplitude (50 uV) and their frequency tended to decline with time through the course of the experiment.

Reversible block of SADA. Addition of d-tubocurarine (10 min; 0.1-0.4 uM; n=8) following the 10 min NEO treatment reduced or abolished SADA. This blockade was partially or fully restored during wash. Decamethonium (1-2 uM; n=6) had a similar action to that of d-TC, but atropine (10 uM; n=3) was without effect.

Backfiring and ACh. In three preparations a small bolus (2.5-4.1 ul, corresponding to a 3-5 sec drug delivery time at 50 ul/min perfusion rate) of 1 M ACh was applied to the preparation via the perfusate, following the 10 min NEO treatment. In these experiments it was observed that ACh produced a transient increase in the incidence of spontaneous backfiring (Figs. 11 and 12), followed by a block of SADA (Fig. 12). Addition of d-tubocurarine (2 uM) following NEO blocked all backfiring and prevented the effects of ACh.

Effect of BOT. Neuromuscular blockade following BOT treatment was confirmed by the virtual absence of miniature end-plate potentials (mepps) and the elimination of end-plate potentials (EPPS) and twitch in response to low frequency indirect stimulation. However, at high stimulus frequencies (50 Hz), sporadic, small amplitude (2-5 mV) EPPS were elicited (Fig. 13A). After the addition of NEO (2 uM), EPPS elicited by this method show a prolongation of

the repolarization phase (Fig. 13C), characteristic of acetylcholinesterase inhibition[46,47].

Stimulus-induced antidromic activity could not be elicited in the phrenic nerves of BOT-blocked preparations upon addition of NEO (2 uM) in a manner similar to controls (Fig. 13D; n=8). Furthermore, spontaneous antidromic activity was never apparent in any of these preparations after the addition of NEO. However, backfiring was induced in the BOT-blocked diaphragms by the infusion of a bolus of ACh (after 10 min NEO treatment; Fig. 14; n=4). It is noteworthy that more recent work in this laboratory utilizing tetanus toxin indicates that exogenously applied ACh is able to elicit backfiring in the absence of NEO.

In conclusion, the experiments reported here demonstrate that NEO is not sufficient by itself to produce SADA in vitro if cholinergic transmission is blocked with BOT. This is observed with concentrations of NEO which are invariably effective in eliciting backfiring in controls. Previous studies have shown that BOT does not prevent the access of the action potential to the nerve terminal[48]. Our observation that sporadic EPPS could be evoked during high frequency stimulation of the nerve confirms this finding in our preparation. Furthermore, BOT appears not to interfere directly with the backfiring mechanism since antidromic activity was observed in BOT-poisoned preparations upon infusion of ACh.

Effect of Calcium Antagonists on Backfiring Calcium antagonists such as verapamil and nifedipine are known to decrease the antidromic activity associated with post-tetanic potentiation in the in situ cat soleus preparation[57]. In an attempt to further understand the mechanistic nature of backfiring in the phrenic nerve, we examined the connection of calcium antagonism in vitro. Further, since organic $Ca^{++}$ antagonists can interfere with spontaneous quantal release of transmitter[58,59], we tested the hypothesis that, like botulinum toxin, organic $Ca^{++}$ antagonists reduce antidromic activity by interfering with spontaneous quantal transmitter release.

The mouse phrenic nerve-hemidiaphragm was prepared for the measurement of antidromic activity as described above. All drugs were dissolved in our standard HEPES-buffered medium and administered vascularly via the cannula.

Antidromic nerve activity was quantified by counting (5001 Universal Counter Timer, Global Specialties Corp., New Haven, Ct) potentials above a set trigger level, usually 50 uV with the aid of a slope/height discriminator (Frederick Haer, Brunswick, ME). Antidromic nerve activity was also monitored aurally (AM 8 audio monitor, Grass, Quincy, MA) and visually on an oscilloscope; these observations were used to assure the accuracy of the count. Neostigmine (2 uM) was administered for 10 min. Antidromic nerve activity was then monitored for 20 minutes after neostigmine was halted. The number of potentials in an 8 min control period (minutes 3-10) were recorded. At the end of minute 10 the preparation was perfused with a second aliquot of control medium with or without 5 uM verapamil, and the number of potentials was recorded during a second period (minutes 13-20). For each preparation, the percent of control activity of the second period was calculated.

For the investigation on spontaneous quantal transmimitter release (mepps), the vascularly perfused mouse phrenic nerve-hemidiaphragm was prepared as for the measurement of antidromic activity, then conventional

intracellular recording techniques were utilized. For one endplate, in each preparation, mepp amplitude, mepp frequency and resting membrane potential were recorded for one minute in control medium and for one minute 3-8 minutes after the start of 5 uM verapamil. Before and after verapamil, the mean amplitude of mepps was determined for two min of recording with a Smartscope II (T.G. Branden, Portland, OR). Mepp frequency was determined by counting the number of mepp's in 1 minute.

Neostigmine bromide and verapamil hydrochloride were purchased from Sigma Chemical Corp., St. Louis, MO. Verapamil was dissolved in control medium in the presence of ethanol, which did not exceed 0.024%. The Students t-test and paired t-test were used as indicated and the null hypothesis rejected if $P < 0.05$.

Following the administration of neostigmine, spontaneous electrical activity was observed along the phrenic nerve. Potentials of 50-100 uV were recorded, sometimes in apparent bursts of up to 4 potentials. Microscopic examination of the diaphragm indicated that this electrical activity was accompanied by the contraction of a few muscle fibers. This antidromic activity was most likely due to action potentials of individual motoneurons which were firing singly or repetitively.

Antidromic activity increased transiently after halting the administration of neostigmine to the preparation. In control medium, (without verapamil), the time-course of antidromic activity following the cessation of neostigmine was such that the total number of potentials in the second recording period was similar to the control period (87%). Similarly, when ethanol was present during the second period (0.024%, as a control for the ethanol vehicle for verapamil), the amount of antidromic nerve activity in the second period was almost identical with that of the control period (96%, S.E.M. = 12, n = 4).

Verapamil (5 uM) significantly decreased antidromic nerve activity. In contrast, verapamil (5 uM) did not decrease either mepp frequency or mepp amplitude (paired t-test); nor did verapamil affect the resting membrane potential; the mean (n = 5) resting membrane potential was -75 mV both before and after verapamil.

We expected verapamil to decrease spontaneous antidromic activity by decreasing spontaneous quantal transmitter release. However, our results are not consistent with this hypothesis. Although we confirmed that verapamil (5 uM) can decrease neostigminie-induced spontaneous antidromic activity, the same concentration of verapamil did not decrease spontaneous quantal transmitter release.

Although we favor the hypothesis that antidromic nerve activity is due to the direct action of acetylcholine on the motor nerve terminals, it has also been proposed that antidromic activity may result from the efflux of $K^+$ from muscle endplates subsequent to the opening of the acetylcholine activated ion channel[60]. If this were true, then verapamil might decrease antidromic activity by a postjunctional antagonism of acetylcholine. In this regard, the observation that the verapamil analogue D600 has been sa non-competive, use-dependent antagonist to iontophoresed acetylcholine[61] is relevant. Additionally, Wachtel[62] observed that verapamil can decreasse average acetylcholine channel life-time, as reflected by a decrease in miniature

endplate current (mepc) decay time. However, verapamil does not decrease mepc decay time in the mouse hemidiaphragm at 5 uM (R.E. Wachtel, College of Medicine, Universitity of Iowa, personal communication). Since verapamil affected neither mepp amplitude nor RMP at the concentration used here, it is unlikely that a channel blocking activity accounts for its antagonism of spontaneous antidromic activity.

Since both verapamil and nifedipine antagonizes antidromic activity[57] and since these compounds are structurally unrelated $Ca^{++}$ antagonists[63], verapamil most likely antagonizes antidromic activity by altering nerve terminal $Ca^{++}$. The observation that decreased $Ca^{++}$ or increased $Mg^{++}$ also antagonizes antidromic activity[64] also supports this conclusion. Evidently, these $Ca^{++}$ antagonists have actions independent of there action on spontaneous quantal transmission which affect motor nerve endings. Both quantal and non-quantal release of acetylcholine could be involved in the genesis of spontaneous antidromic activity and verapamil selectively affects non-quantal release at the concentration studied here. However, this is unlikely. Alternatively, verapamil may be acting at a site which decreases nerve excitability. This latter possibility is consistent with the finding that the $Ca^{++}$ antagonist diltiazem decreased the amplitude of spinal action potentials elicited by antidromic stimulation of frog ventral roots[65]. Thus, backfiring may be related to modulation of the depolarized state of the nerve terminal, i.e., excitability. Whether the putative prejunctional nicotinic feedback receptor is linked to the verapamil site remains to be determined.

**G. Subchronic <u>in vivo</u> exposure to DFP - effect on negative modulation of ACh release**

DFP has been shown to reduce the FC in a manner similar to those of NEO and physostigmine via a frequency-dependent mechanism which we believe involves an indirect effect on a prejunctional cholinoceptor. That is, even partial inhibition of AChE at the neuromuscular junction allows the cleft levels of residual ACh to increase after each nerve stimulation. If the frequency of stimulation is high enough (5Hz and above), the residual ACh in the absence of AChE activity reaches an effective concentration for stimulating, in theory, the prejunctional ACh autoreceptors. Negative feedback would result so that the next volley of nerve stimulation would fail to release the normal amount of ACh, and so on. Our most recent FC data indicate that negative feedback is present in the isolated hemidiaphragm preparations from rats treated subchronically (1, 3 and 5 days) with sublethal doses of DFP. Atropine was administered with each DFP injection as a precaution against post-injection cholinergic distress. Negative feedback was present without the addition of any other compound and is antagonized by dTC ($10^{-8}$). Negative feedback disappeared if animals were allowed to recover for 48 hrs prior to isolation of the hemidiaphragm. This is an important observation for indicating that moderate, subchronic exposure to an organophosphate agent alters neuromuscular transmission despite the absence of gross motor deficits in the animal. These alterations could lead to a compromise of neuromuscular function under conditions of mild stress or exertion.

**H. Chemiluminescence method for measuring picomolar amounts of ACh in perfusate from VPRH.**

Release of endogenous acetylcholine (ACh) from the isolated, anticholinesterase-treated vascular perfused rat phrenic nerve-hemidiaphragm (VPRH) is approximately 1 pmol/min at rest and not more than 20-30 pmol/min during maximum nerve stimulation[3]. A highly sensitive and specific extraction/assay procedure is required to detect small changes in this ACh release due to pharmacological or toxicological challenges. We have explored a chemiluminescent assay (CLA) as an alternative to the previously established radioenzymatic assay (REA)[49] in view of the potential hazards of radioactivity ($^{32}P$), and the unpredictability and high cost of the REA.

A chemiluminescent assay for ACh was first successfully applied to brain and muscle extracts[50]. Three critical reactions are involved in this assay procedure: first, ACh in an aqueous sample is converted to choline (Ch) by acetylcholinesterase (AChE; EC 3.1.1.7); second, Ch is converted to betaine and $H_2O_2$ by choline oxidase (ChOx; EC 1.1.3.1.7); and finally, $H_2O_2$ is reacted with luminol and horseradish peroxidase (HRP; EC 1.11.1.7) to generate the chemiluminescence which is electronically measured as an indicator of the amount of ACh originally in the sample.

Häggblad et al.[51] modified the original CLA method of Israël and Lesbats[50] to measure $K^+$-evoked release of ACh from a rat hemidiaphragm preparation. We found this chemiluminescent method to be inadequate for our pharmacological studies on nerve-evoked release of ACh from the VPRH. The use of dilute hydrochloric acid solutions, as well as substances in the perfusate not removed by their extraction procedure, interfered with the CLA under our conditions[52].

We have modified the procedures of Häggblad et al.[51] in order to measure nerve-evoked release of ACh from the VPRH. Further, to assure the specificity and accuracy of this new CLA, we have directly compared results obtained from the CLA to those from the REA routinely used for this preparation. Our new method and results are discussed below.

In brief, the left hemidiaphragms from male Long-Evans hooded rats (Simonsen Laboratories, Inc., Gilroy, CA) weighing 300-440g were prepared for vascular perfusion[3,43] with a HEPES-buffered medium (pH 7.4) containing in mM: 116 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1.0 $MgCl_2$, 11.0 glucose, 6.0 HEPES, 0.010 Ch chloride and 0.010 neostigmine to inhibit the hydrolysis of ACh by endogenous AChE. Perfusate was collected from the VPRH continuously in 30 minute periods, amounting to approximately 1.1 ml of perfusate per period. The preparation was at rest (unstimulated) for the first 30 minute period and then stimulated indirectly (1-2 V, supramaximal; 0.2 ms rectangular pulses to the phrenic nerve via a Grass Instruments SD9 Stimulator) at 10 Hz for 3 hours to give a total of 7 samples. The perfusate samples were centrifuged for 10 min, at 1000Xg and 4°C. In order to determine the amount of ACh released during stimulation, aliquots of samples 2 through 6 were taken for both the periodide precipitation-extraction/CLA (2 x 125 ul per sample as duplicates) and the tetraphenylboron extraction/REA (2 X 250ul per sample). Calibration of the assays was achieved by adding standard amounts of ACh (0, 30, 60, 90 pmol) to pooled perfusate (samples 1 and 7, and the remainder of samples 2 through 6).

The tetraphenylboron extraction/REA was performed by the method of Goldberg and McCaman[53] as modified by Bierkamper and Goldberg[49] for the VPRH. This REA method was used exclusively in the early phases of this contract[1,2].

In preparation for the CLA, the periodide precipitation-extraction procedure of Häggblad et al.[51] was used with the following modifications. A 0.4M sodium acetate buffered solution (pH = 4.0) was used in place of glass distilled water to dilute the sample aliquots prior to the periodide precipitation step. This substitution improved luminescence. The improvement was not simply a pH effect since luminescence values for extracted ACh samples did not vary with the pH of the sodium acetate solution (pH 3 to 7). We avoided the use of tritium-labelled ACh as an internal standard as it contributed to luminescence. Tetramethylammonium chloride (6 mg/ml) was substituted for the bromide. Dilute formic acid ($10^{-3}$M; 400ul) was used in place of dilute hydrochloric acid to free ACh and Ch from their complexes with periodide, washing with 2 ml of anhydrous diethyl ether. Additionally, it was discovered that the ether used for washing must be peroxide free (tested by the procedure of Stewart and Young[54]). Extracted samples were capped and stored in the dark at 0° for 12-16 hours.

Prior to the assay the extracted perfusate samples were first thawed, uncapped and placed in an open dark area for 1 hour and then recapped. It was crucial to uncap the thawed samples to allow the last traces of iodine and ether to evaporate. At all times during the assay the extracted perfusate samples were kept on ice in the dark.

The following luminol-enzyme reagent was used for the CLA: 165 ul of ChOx (100U/ml) in 4.01 ml of 0.2 M sodium phosphate buffer (pH 8.6), 35 ul of HRP (1 mg/ml), and 170 ul of 1 mM luminol. All of the reagents and samples were kept on ice.

An aliquot (100 ul) of each extracted perfusate sample was added to a polypropylene tube (8 X 50mm). The tubes must be handled with latex gloves because fingerprints contribute to luminescence. Each tube was placed in the reaction well of the luminometer (Turner model 20e, thermostated to 30°). Luminol-enzyme reagent (200 ul) was injected, briskly without splashing, into the sample to start the reaction with choline. [The resulting integrated luminescence of this reaction may be used to quantify choline if desired.] After the chemiluminescence from the reaction had decayed to a stable baseline (8 - 10 min), the endogenous ACh in the sample was converted to choline by the injection of AChE (100 ul, 12.5 U/ml). The integrated luminescence value for 20 s of chemiluminescent reaction of the choline liberated from ACh with luminol-enzyme reagent was used to calculate the amount of endogenous ACh in each sample.

A second series of experiments was designed to determine whether certain cholinergic drugs interfere with the extraction assay procedure. Perfusate was collected continuously from the VPRH for 3.5h (30 minutes rest, 3h at 10 Hz), pooled, and then centrifuged. Aliquots (500 ul) of the perfusate were treated with 5 ul aqueous stock solutions ($10^{-3}$M) of drug to give final concentrations of $10^{-5}$M. A 500 ul aliquot of untreated perfusate containing only released, endogenous ACh served as the control. These samples were extracted and assayed by CLA as described above.

Our modified CLA procedure provides an accurate and reliable measure of endogenous ACh released by indirect stimulation of the VPRH. This was demonstrated by a direct comparison of the CLA and REA measurements of ACh released over time (Fig. 15). A two-way analysis of variance showed that the results from the CLA did not differ from those of the REA. Both the

tetraphenylboron extraction/REA and the periodide extraction/CLA methods yielded limits of sensitivity of approximately 3 pmol as determined by extracted ACh standards. Standard curves typically had correlation coefficients (by linear regression analysis) of between 0.95-0.98 for the CLA in the range of 2 to 40 pmol. Further, Fig. 15 indicates that there is less variability in values from the CLA than from the REA. Thus, the CLA yields a more consistent pattern of ACh release over time.

Additional experiments were performed to determine if a number of frequently used cholinergic drugs ($10^{-5}$M) altered the luminescence of extracted perfusate. These drugs included atropine, hexamethonium bromide, decamethonium bromide, aminopyridine, aBGT, DFP, nicotine, carnitine, dTC, physostigmine and carbachol. A one-way analysis of variance of these data indicated a lack of interference by these drugs. We have found that dTC and physostigmine interfere with the CLA under some circumstances. However, these drugs can be removed chromatographically; dTC by the method of Fletcher and Forrester[55] and physostigmine by the method of Molenaar and Polak[56].

The CLA has a number of advantages over the REA. It has been our experience that about one VPRH in ten fails to release normal levels of ACh due to a variety of reasons (e.g., nerve damage during dissection)[3]. Although it has not been possible to detect a failed preparation until completion of the REA, the CLA detects problems after running only a few samples, thus saving considerable technician time. Moreover, less sample is required for the CLA than the REA so that an assay may be repeated, if necessary. In addition, the time to run the CLA can be halved by pre-reacting the samples en masse with the luminol-enzyme reagent to remove the choline prior to measuring the ACh. This has been adopted as a routine procedure in our laboratory since it saves time and reduces variability even further. The cost of both set-up and routine operation is less for the CLA than the REA. Reagent cost is lower for the CLA when considering the expense of the radioactive compound, scintillation cocktail and vials, radioactive waste disposal and service contracts on the scintillation counter for the REA. Thus, the CLA is more economical than the REA.

In conclusion, the CLA procedure described in this report provides a method for analysis of ACh in perfusate collected from the rat hemidiaphragm preparation and yields results similar to those achieved with the REA, but at reduced cost and with minimal cholinergic drug interference. Therefore, this method has totally replaced the use of the REA in this laboratory.

## IV. Discussion

Local mechanisms in the motor nerve terminal which may regulate the amount of ACh release per nerve impulse have been the focus of this year's experimental effort. We have been seeking the relative importance of these mechanisms under varying conditions of nerve firing and especially in the presence of cholinergic drugs and anticholinesterase agents. Our goal has been to determine the normal function of the mechanisms of regulation and then to study their function under conditions which mimic disease states, demanding physiological situations and conditions of neuromuscular toxicity.

The hypothesis that ACh release is regulated by a prejunctional cholinoceptor has been a predominant area of investigation in our laboratory.

We find that cholinergic (nicotinic) agonists lower the amount of ACh released during nerve stimulation. The depression in ACh release is substantial enough to decrease the force of contraction in the performing muscle. The ability of low doses of curare to antagonize the agonist-induced decrease in ACh release lends support to the theory that a prejunctional cholinergic recognition site, a nicotinic receptor, is involved. However, other mechanisms, such as $K^+$ release and depolarization block, must be addressed as well. Our backfiring studies do not support strongly a substantial influence of $K^+$ in a negative feedback scheme within reasonable physiological bounds. Intracellular recordings do not unveil depolarization block as a predominant mechanism although it is occasionally and unpredictably observed. Receptor desensitization must be also be considered as an explanation for depresses neuromuscular transmission during elevated agonist conditions in the junctional cleft. There is little doubt that ACh receptor desensitization occurs during longterm exposure to residual agonist in the cleft. However, we conclude from our studies to date that postjunctional receptor desensitization does not account for the negative feedback observed during agonist's presence. This was confirmed by intracellular recordings, measurement of FC, and by actually measuring the release of ACh by biochemical assay.

The nicotinic antagonists dTC ($10^{-6}$M) and aBGT have been shown to increase the stimulated release of ACh and to antagonize the effects of nicotinic agonists on ACh release. However, these agents do not appear to enhance the normal force of contraction of the muscle. To the contrary, aBGT actually blocks muscular contraction by "irreversibly" blocking the postjunctional nicotinic receptors; curare has similar properties at higher doses ($10^{-4}$M). Low concentrations of dTC block backfiring, agonist-induced negative feedback and yield frequency-dependent neuromuscular blockade consistent with a prejunctional action. Our results suggest that dTC acts via a prejunctional cholinoceptor to modulate ACh release. Further details on the action of dTC are presented below.

ACh, the natural agonist, has been shown to have a prejunctional action which is frequency- and dose-dependent. We have discovered that stimulation of the nerve must reach 5Hz before significant negative feedback occurs in the presence of partial AChE inhibition. We take this to mean that the interval between firing must be short enough to induce the accumulation of ACh in the junctional cleft. This negative feedback affects muscular performance and is antagonized by low doses of dTC ($10^{-8}$). Subchronic exposure to AChE inhibitors results in a residual enhanced negative feedback in the phrenic nerves of isolated VPRH. ACh perfused into the cannulated vascular system of the mouse hemidiaphragm results in transient backfiring of the phrenic nerve. Intracellular recordings in the presence of dTC suggest a prejunctional action where ACh feeds back on the nerve terminal; dTC antagonizes this response in a frequency- and dose-dependent manner. Therefore, ACh undoubtedly exerts a prejunctional action which appears to regulate the amount of ACh released on subsequent stimuli. However, the degreee of contribution to regulation of ACh under non-stressed in vivo physiological states is uncertain. Nonetheless, it is reasonable to assume that negative feedback becomes operative and significant during systemic anti-AChE intoxication.

A quantitative autoradiographic technique has demonstrated that $^{125}$I-alpha bungarotoxin binding sites are transported in sciatic nerve in an orthograde and retrograde manner. We postulate that these binding sites represent cholinoceptors destined to become, in part, prejunctional nicotinic

receptors. These studies have been completed this year and the technique expanded to evaluate the effects of various toxic agents on axonal transport of the binding sites. The methodology appears to be valuable in the study of the neuropathology of certain neurotoxicants with regard to the axonal transport of specific protein substances. Future studies may profit from examining negative feedback in the early stages of a chemically-induced peripheral neuropathy when it has been determined that the axonal transport of the binding sites is compromised.

The effect of choline in ACh synthesis and release has been investigated. We have shown previously that prevention of choline uptake by the nerve terminal with hemicholinium (HC-3) induces a decline in stimulated ACh release; i.e., ACh synthesis cannot keep pace with demand. Moreover, elevated choline concentrations (30-60mM) appear by mass action to raise stimulated ACh release from isolated nerve-muscle preparations; i.e., ACh synthesis is enhanced. Furthermore, the rate-limiting step for ACh synthesis appears to be the availability of choline in the nerve terminal. Thus, we have been interested in the role choline plays in negative feedback and in the general modulation of ACh release. Our experiments have show .that choline (up to 60mM) has no effect on negative feedback induced by NEO in the FC studies. Under normal physiological conditions, choline seems to be in excess. However, in the presence of DFP or certain chemical substances such as HC-3, choline availability decreases and results in depletion of releasable ACh during high frequency stimulation. This has been demonstrated in the choline efflux studies from VPRH. Neurotoxic organophosphate agents reduce the efflux of choline from the preparation and have a negative influence on ACh release. The non-neurotoxic AChE inhibitors, physostigmine and paraoxon do not alter choline efflux or change ACh released. We speculate that DFP and TOCP may interfere with phospholipid metabolism such that choline is not made availabe from its phosphatidyl choline stores. We conclude from our studies that the negative feedback, which is presumably due to the putative prejunctional cholinoceptor, cannot be through a choline supply problem. The negative feedback occurs in milliseconds rather than minutes, as would be necessary for a depletion of stores through blockade of ACh synthesis. Thus, we are dealing with two separate but important mechanisms for modulating ACh release. It is possible that during exposure to certain neurotoxic anticholinesterase agents both mechanisms surface; choline availability at the nerve terminal may decrease and residual ACh in the cleft may increase. Both conditions would decrease the amount of ACh released during stimulation and may have a negative influence on neuromuscular transmission, resulting in muscular weakness.

The origin of motor nerve antidromic activity (backfiring) induced by anticholinesterase treatment was examined in the mouse phrenic nerve-hemidiaphragm preparation. Botulinum toxin was used to determine whether backfiring is due to a) a direct effect of the cholinesterase inhibitor on the nerve terminal, or b) an indirect effect via the prolongation of the action of acetylcholine. In previously untreated control preparations, neostigmine produced spontaneous and stimulus-induced antidromic activity in the phrenic nerve when rapidly introduced into the diaphragm via its vasculature. This activity could be reversibly blocked by d-tubocurarine and decamethonium, but not by atropine. Neostigmine-induced backfiring did not occur in preparations in which transmitter release was blocked with botulinum toxin. Infusion of a small bolus of a high concentration of acetylcholine following neostigmine treatment resulted in a short-term increase in the incidence of antidromic activity, followed by block, in both controls and botulinum toxin-

treated preparations. It is concluded that transmitter release is necessary for the production of backfiring following cholinesterase inhibition since neostigmine alone does not elicit antidromic activity in botulinum toxin-treated preparations at concentrations which are effective in controls. Our results support the hypothesis that the effects of neostigmine on the motoneuron terminal are mediated by the prolonged action of acetylcholine that occurs with inhibition of acetylcholinesterase.

The studies on antidromic firing lend support to the presence of cholinergic recognition sites on motor nerves. These data are supported as well by the $^{125}$I-aBGT binding sites found to be transported in sciatic nerve. Although backfiring per se is not generally viewed as a normal physiological means of regulating ACh release, it may play a role in abnormal conditions such as anti-AChE poisoning. Retrograde action potentials would collide with incoming impulses and prevent depolarization of the nerve terminal; i.e., this would prevent ACh release. On the other hand, backfiring could occur in one branch of a motor unit and spread orthodromically at branch points to other branches. This, in effect, would increase ACh release and perhaps fasciculations within the muscle bundle. These studies also promote the consideration of older theories concerned with the role of $K^+$ in the environment surrounding the nerve terminal. It is believed by some investigators[15,16] that sADA may be caused by excessive $K^+$ release (by muscle cells?) so that the nerve terminal would become depolarized and activate the first node of Ranvier. This would evoke a retrograde action potential. This alernative theory will also be addresed in our future studies.

The calcium antagonist verapamil decreased spontaneous, neostigmine-induced backfiring implicating the need for calcium in the process. It is likely that calcium either plays a role in the amount of ACh released or modifies the membrane potential (and thus excitability) of the nerve terminal or both. This would influence backfiring as we have shown experimentally. Whether a calcium mechanism is directly linked to the putative prejunctional nicotinc cholinoceptor is uncertain.

Additional studies with calmodulin inhibitors lend further support to the role of calcium in modulating ACh release. A test of the hypothesis based on the pathway illustrated in Fig. 4 confirms that ACh release can be modified by antagonizing $Ca^{++}$-calmodulin. Experiments with theophylline and forskolin suggest that calmodulin is linked to cAMP which, via a protein kinase, ultimately modifies ACh release. Stimulus-induced calcium entry therefore plays a role in neurotransmitter release. It is conceivable that the prejunctional cholinoceptor is linked to the control of calcium entry since we have shown that the negative feedback is too fast for a mechanism involving ACh synthesis. Whether this link is via the calmodulin pathway is uncertain. Taken together with the backfiring results, these data strongly support the hypothesis that a local calcium regulatory mechanism modulates ACh release.

A chemiluminescent assay coupled to a periodide extraction method is described for the measurement of ACh release from the vascularly perfused rat phrenic nerve-hemidiaphragm preparation. A direct comparison of the CLA with an established radioenzymatic assay for ACh demonstrates that the two assays are quantitatively similar and yield equivalent limits of sensitivity of approximately 3 pmol. The periodide extraction/CLA method is routinely less variable and more reliable than the tetraphenylboron extraction/REA method. Cholinergic drug interference with the CLA is minimal. The absence of

radioactivity and the reduced cost of the CLA make it an attractive alternative to the REA. Consequently, the CLA method has replaced the REA method in this laboratory.

In conclusion, this investigation clearly demonstrates that ACh release can be modulated by local prejunctional mechanisms. These mechanisms include a prejunctional nicotinic cholinoceptor, precusor availability and probably nerve terminal excitability as modified by ionic channel activity at the membrane level. All of these mechanisms are disturbed by AChE inhibition by neurotoxic anticholinesterase agents, resulting in a compromise in neuromuscular transmission and, therefore, performance.

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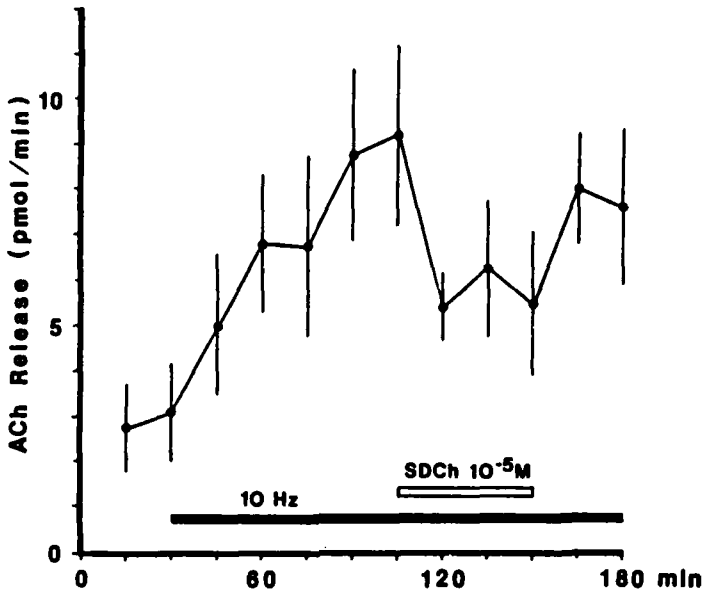


Figure 1. Effect of suberyldicholine on ACh release from VPRH. ACh release was measured by radioenzymatic assay in perfusate samples collected every 15 min. Neostigmine ($10^{-5}$M) allowed complete recovery of released ACh at rest or during 10 Hz phrenic nerve stimulation. Suberyldicholine (SDCh; $10^{-5}$M), administered via the cannulated vascular system, significantly depressed ACh release at 135-150 min [$P(t) < 0.05$; $n=5$]. Washout of suberyldicholine restored release to normal limits.

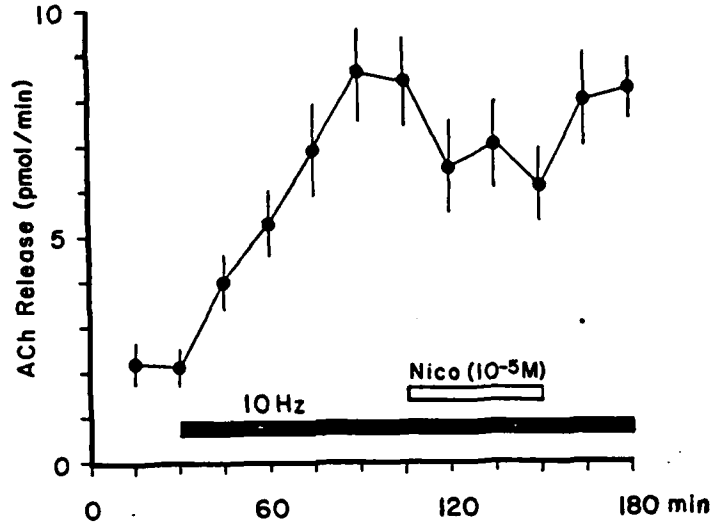


Figure 2. Effect of nicotine on ACh release from VPRH. ACh release as measured by radioenzymatic assay in perfusate samples collected every 15 min. Nicotine (Nico; $10^{-5}$M), administered via the cannulated vascular system, significantly depressed stimulated (10Hz) ACh release at 135-150 min [$P(t) < 0.05$; n=6]. Washout of nicotine restored release (180min) to pre-drug levels (90-105 min).

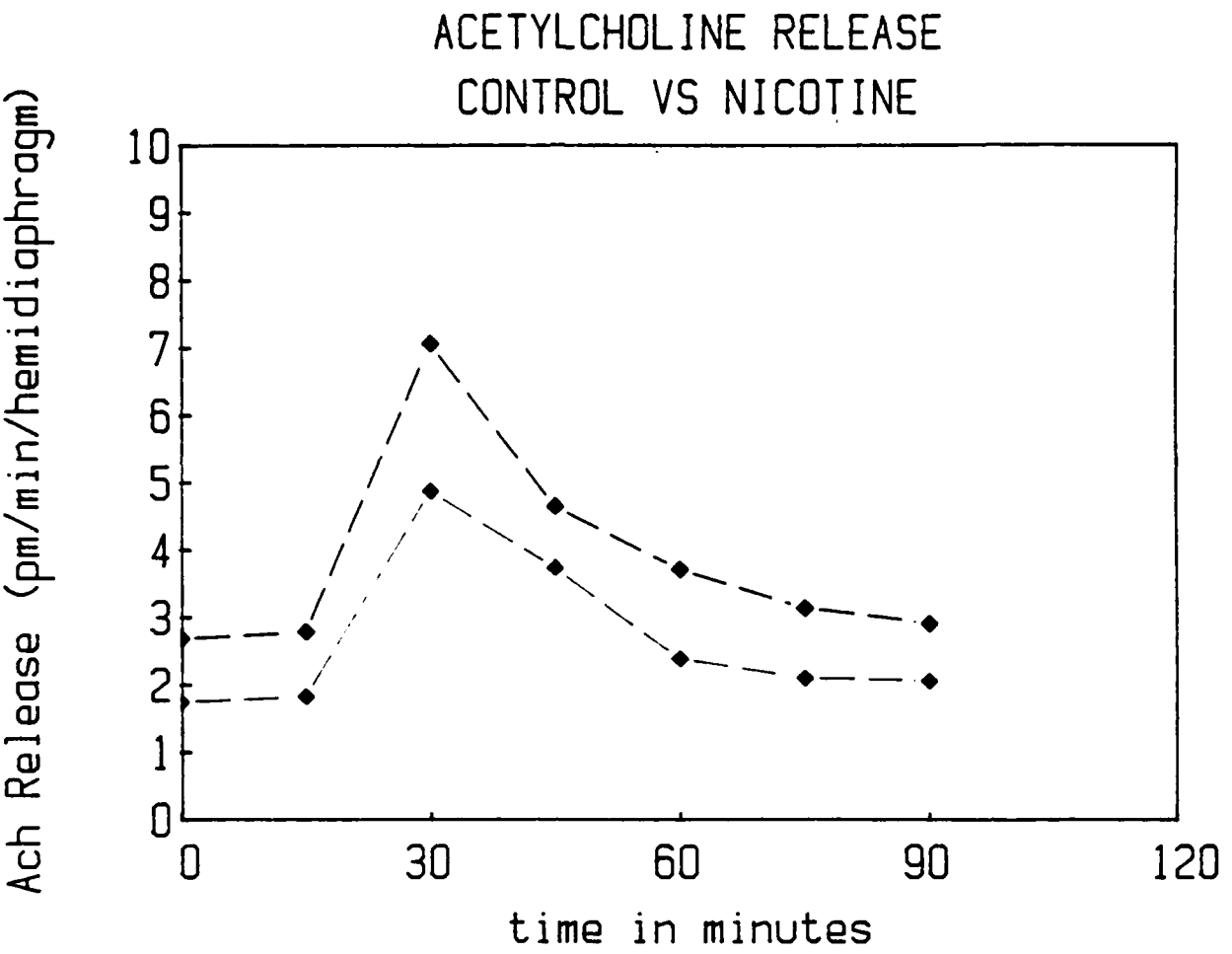


Figure 3. Nicotine alters ACh release as measured by chemiluminescent assay. ACh was measured in perfusate collected every 15 min. Nicotine ($10^{-5}$M) was perfused into the cannulated vascular during the entire experiment in 5 VPRH preparations (lower curve). Five control experiments (upper curve) using an identical protocol are presented for comparison. Stimulation of the phrenic nerve was for one 15 period only beginning at 30 min after the collection of two resting samples. The standard errors were less than 20% and are not presented for clarity. The two curves are not statistically significantly different by Student's t test.

## Hypothetical Pathway

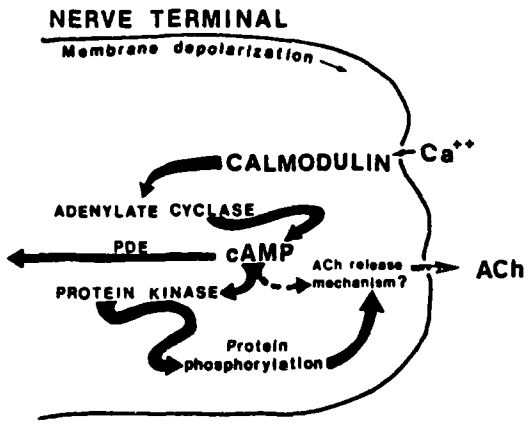


Figure 4. Hypothetical pathway of $Ca^{++}$-calmodulin regulation of ACh release.

Fig. 5

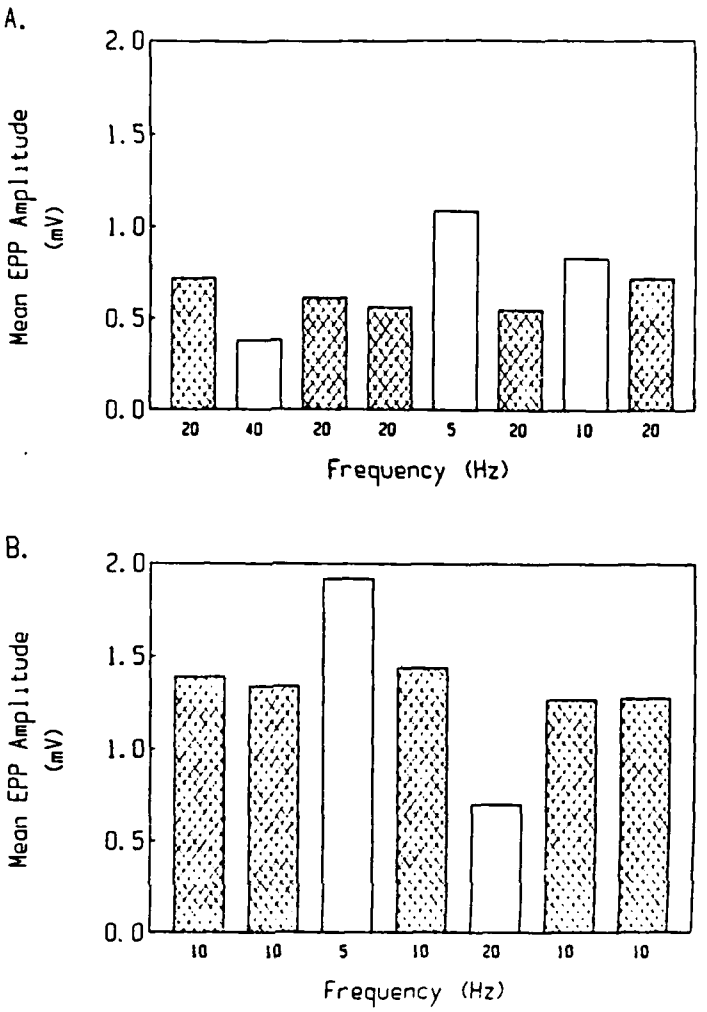


Figure 5. Mean EPP amplitudes at different frequencies during continuous stimulation at holding frequencies 20 Hz (**A**) and 10 Hz (**B**) in the presence of $5 \times 10^{-7}$ M dTC. For each holding frequency, mean EPP amplitudes were determined from a single cell at 3 minute intervals. At each frequency, 40 EPPs were sampled and averaged. All SEMs were less than 0.02 mV and are omitted for clarity. Cross-hatched bars represent amplitudes at the holding frequency.

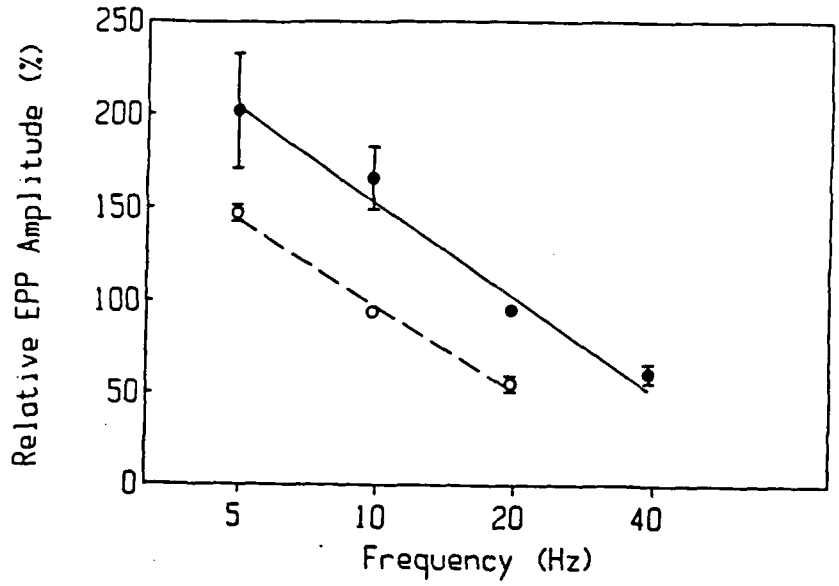


Figure 6. Relative EPP amplitudes versus log of stimulation frequency in the presence of $5 \times 10^{-7}$ M dTC. For each cell, mean EPP amplitude at each frequency was expressed as a percentage of the mean EPP amplitude at the holding frequency of the immediately preceding period.
Solid regression line represents 20 Hz holding frequency. Each point is the mean $\pm$SEM of 6 or more observations from 9 cells in 7 preparations. SEM at 20 Hz was smaller than the symbol.
Dashed regression line represents 10 Hz holding frequency. Each point is the mean $\pm$SEM of 5 or more observations from 7 cells in 4 preparations. SEM at 10 Hz was smaller than the symbol.

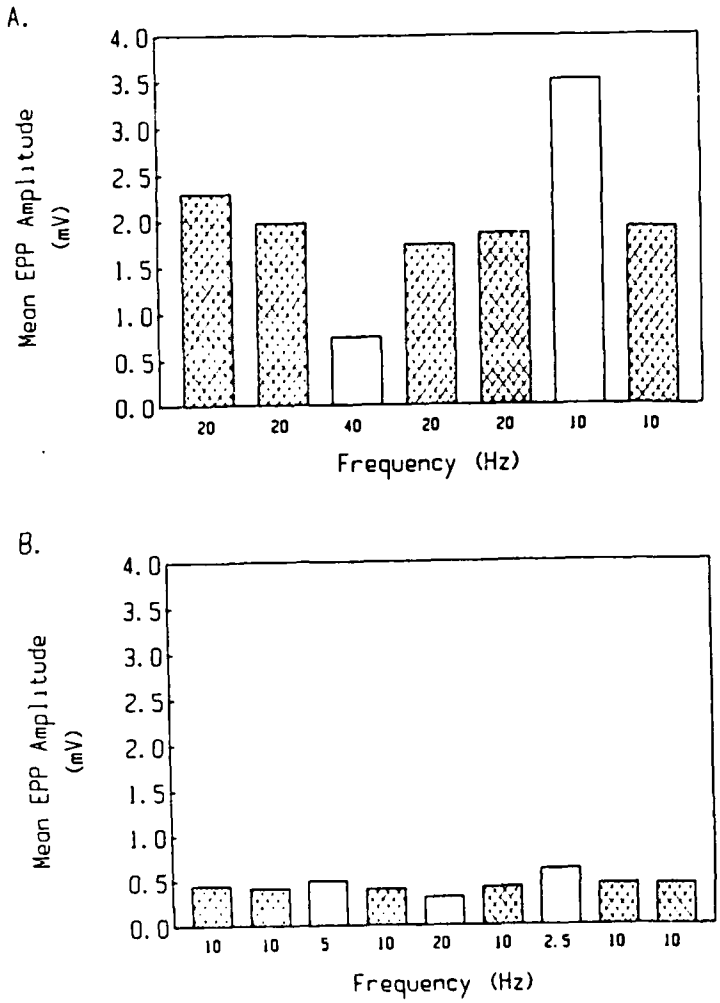


Figure 7. Mean EPP amplitudes at different frequencies during continuous stimulation at $2.5 \times 10^{-7}$M (A) and $1.5 \times 10^{-6}$M dTC (B). For each concentration of dTC, mean EPP amplitudes were determined from a single cell at 3 minute intervals. At each frequency, 40 EPPs were sampled and averaged. SEMs were small (less than 0.05 mV at $2.5 \times 10^{-7}$, less than 0.015 mV at $1.5 \times 10^{-6}$) and omitted for clarity. Cross-hatched bars represent amplitudes at the holding frequency.

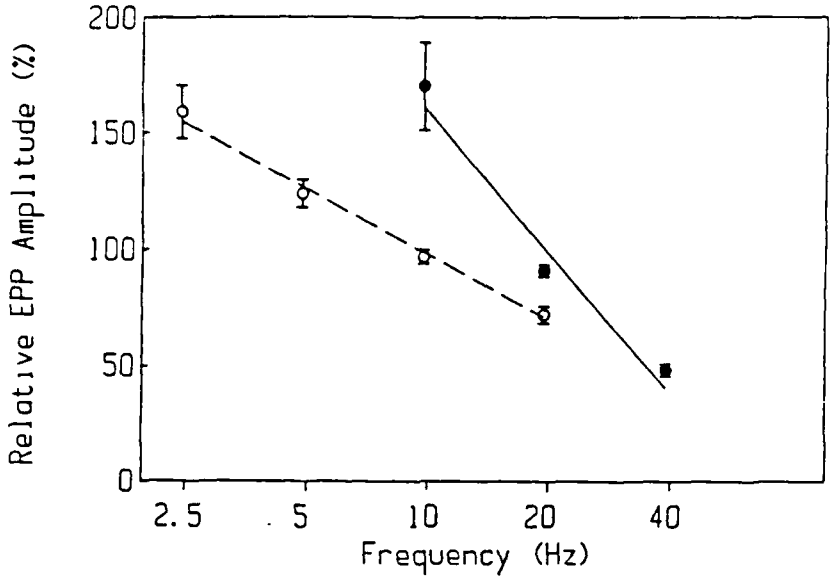


Figure 8. Relative EPP amplitudes versus log of stimulation frequency in the presence of a low and a high concentration of dTC. For each cell, mean EPP amplitude at each frequency was expressed as a percentage of the mean EPP amplitude at the holding frequency of the immediately preceding period. Solid regression line represents $2.5 \times 10^{-7}$M dTC, 20 Hz holding frequency. Each point is the mean $\pm$SEM of 5 or more observations from 8 cells in 5 preparations. Dashed regression line represents $1.5 \times 10^{-6}$M dTC, 10 Hz holding frequency. Each point is the mean $\pm$SEM of 4 or more observations from 8 cells in 5 preparations.

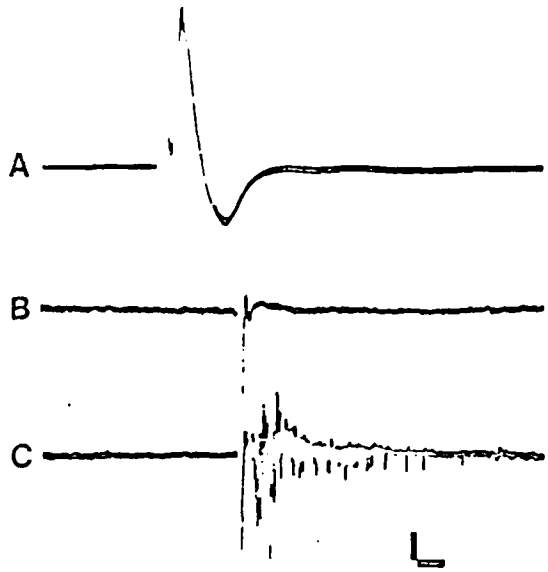


Figure 9. Effects of neostigmine on response recorded from the phrenic nerve following a single nerve stimulus in the mouse diaphragm preparation. A-B, orthodromic action potentials recorded from the phrenic nerve following repeated (A) or single (B) stimuli; C, response recorded from the same phrenic nerve following a single stimulus one minute after addition of neostigmine (2 uM) via the vasculature of the diaphragm. Vertical bar: 0.50 mV (A); 0.05 mV (B&C); horizontal bar: 0.50 msec (A), 10 msec (B&C).

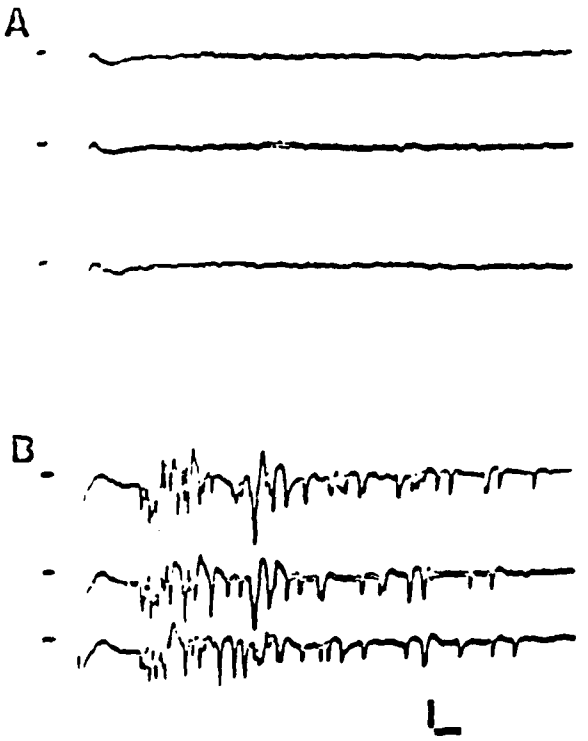


Figure 10. Effect of neostigmine on responses recorded from the mouse phrenic nerve following 3 succesive stimuli 10 sec apart. A, control responses recorded from the phrenic nerve. Orthodromic volleys and stimulus artefacts are off scale. B, responses recorded from the same phrenic nerve during wash period (see Methods) following addition of neostigmine (2 uM) for 10 min. Vertical bar: 0.10 mV; horizontal bar: 2.5 msec.

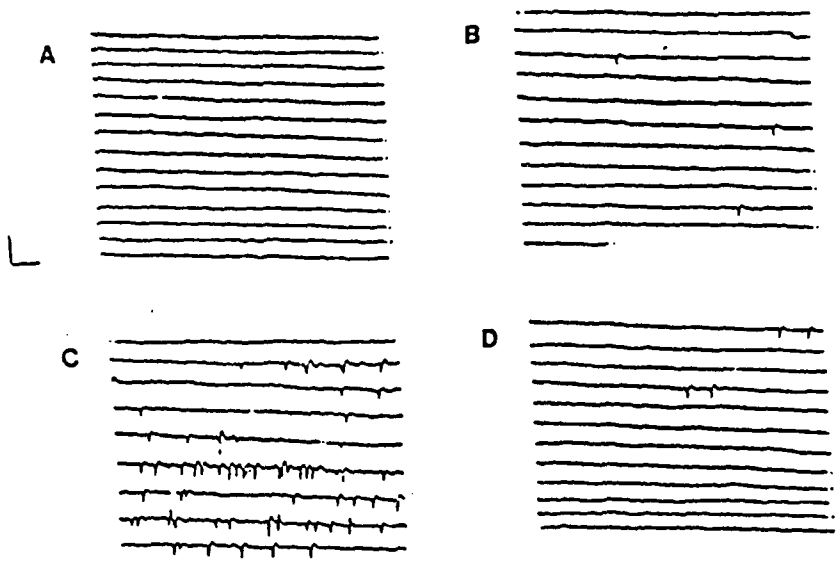


Figure 11. Effect of acetylcholine on non-stimulus associated backfiring produced by neostigmine in the mouse phrenic nerve-diaphragm preparation. A, transient background recordings from the phrenic nerve. B, transient recordings from the same phrenic nerve 2 min after addition of neostigmine through the vasculature of the diaphragm. Neostigmine was continued to be added for a total of 10 min. C, response recorded from the same nerve 5 sec after addition of 2.5 ul of acetylcholine (1 M) via the central diaphragmatic vein. D, transient recording of activity in the same phrenic nerve following 5 min of wash after the addition of acetylcholine. Vertical bar: 0.20 mV; horizontal bar: 5 msec.

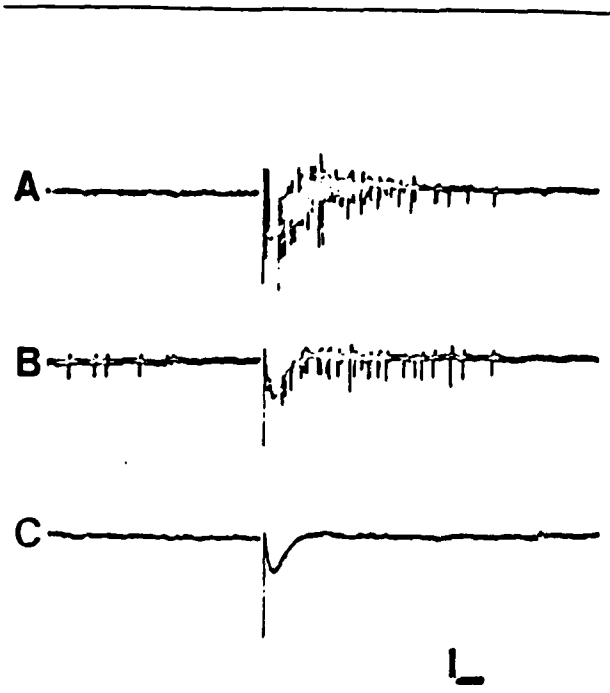


Figure 12. Effect of acetylcholine on stimulus-induced antidromic activity produced by neostigmine in the mouse phrenic nerve-hemidiaphragm preparation. A. Response recorded from the phrenic nerve following a single stimulus 2 min after addition of neostigmine (2 uM) via the diaphragm vasculature; B. Response recorded from the same phrenic nerve following a single stimulus 5 sec after infusion of 4.0 ul acetylcholine (1M) via the diaphragmatic vein; C. Response recorded from the same phrenic nerve following a single stimulus 10 sec after infusion of 4.0 ul acetylcholine (1M). Vertical bar: 0.10 mV; horizontal bar: 10 msec.

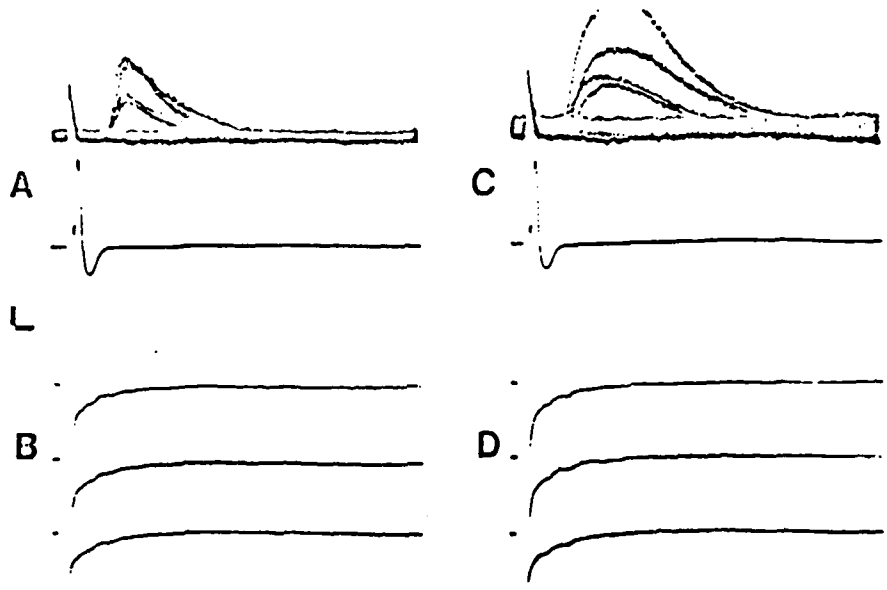


Figure 13. Effect of Botulinum toxin treatment on responses recorded from the mouse phrenic nerve after infusion of neostigmine through the diaphragm vasculature. A, upper trace, intracellular responses recorded from a botulinum toxin-treated mouse diaphragm end-plate region following supramaximal stimulation of the phrenic nerve at a frequency of 50Hz; lower trace, responses recorded from the same phrenic nerve stimulated above. B, responses recorded from the same phrenic nerve during 3 succesive stimuli 10 sec apart. C, same as in A, 2 min after addition of neostigmine (2 uM) via the diaphragm vasculature. D, same as in B, 4 min after addition of neostigmine (2 uM). Neostigmine was added for a total of 10 min. Vertical scale: 1 mV (A & C), 0.10 mV (B & D); horizontal scale: 1 msec (A & C), 2.5 msec (B & D).

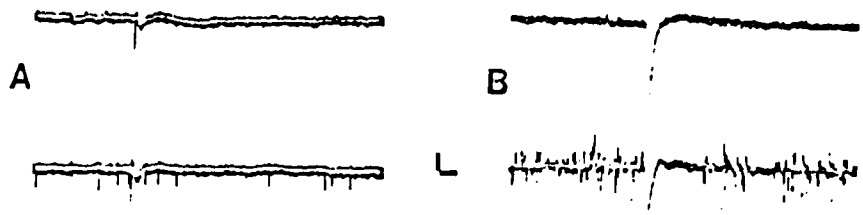


Figure 14. Effect of acetylcholine on responses recorded from mouse phrenic nerves in botulinum toxin-treated phrenic nerve-diaphragm preparations. A, upper trace, response recorded from phrenic nerve of a botulinum toxin-treated preparation following a single nerve stimulus after addition of neostigmine (2 uM, neostigmine added for a total of 10 min) ; lower trace, response recorded from same nerve following a single nerve stimulus 5 sec after addition of 4 ul of acetylcholine (1 M) through the diaphragm vasculature. B, upper trace, response recorded from another phrenic nerve of a botulinum toxin-treated preparation following a single nerve stimulus after addition of neostigmine (2uM, added for a total of 10 min); lower trace, response recorded from same nerve following a single nerve volley 5 sec after addition of acetylcholine as in A. Vertical scale: 0.025 mV; horizontal scale: 25 msec (A), 5 msec (B).

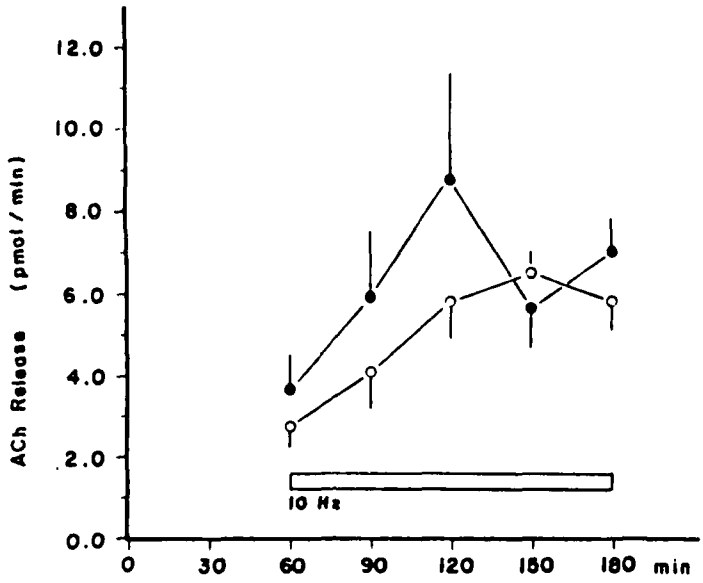


Figure 15. Stimulated (10 Hz; open bar) release of ACh from the vascular perfused rat hemidiaphragm preparation determined by split perfusate samples using the chemiluminescent assay (open circle) and the radioenzymatic assay (solid circle). The values are the means ± S.E.M. for five preparations. A two-way analysis of variance for acetylcholine release shows no significant difference between assays ($p > 0.05$). NEO ($10^{-5}$M) and Ch (10 uM) were present in the perfusion medium.

TABLE 1: Force of contraction comparisons

| Treatment | Conc. | n | %FC (Indirect) / %FC (Direct) |
|---|---|---|---|
| CONTROL | | 3 | 77.0 ± 9.2 / 72.0 ± 8.9 |
| FLU | $10^{-5}$ M | 4 | 31.8 ± 2.9** / 60.5 ± 1.8 |
| FLU + THEO | $10^{-5}$ + $10^{-3}$ M | 3 | 41.7 ± 11.9 / 67.3 ± 9.4 |
| FLU + THEO | $10^{-5}$ + $10^{-3}$ M | 2 | 71.4 / 75.6 |
| CPZ | $10^{-5}$ M | 3 | 25.3 ± 0.3** / 67.7 ± 5.0 |
| CPZ + THEO | $10^{-5}$ + $10^{-3}$ M | 2 | 50.0 / 81.9 |
| CPZ + THEO | $10^{-5}$ + $10^{-3}$ M | 2 | 77.9 / 79.2 |
| TFP | $10^{-5}$ M | 3 | 43.0 ± 5.1* / 57.0 ± 5.6 |
| W-7 | $10^{-5}$ M | 3 | 41.4 ± 15.6 / 53.0 ± 17.5 |

Force of contraction at 60-70 min is presented as %FC ± SEM of control FC (100%) recorded after 30 min of equilibration in each preparation (n). Normalized %FC of treatment groups is compared to %FC of untreated control VPRH subjected to an identical stimulation paradigm (0.5 Hz; direct and indirect; 10 s; 50 Hz). FC decreased by approximately 25% between 30 and 60 min as a result of episodes of higher stimulation frequencies, thus depleting more of the bioenergetic capacity of the VPRH than would be expected at only 0.5 Hz stimulation. %FC in the presence of fluphenazine (FLU), chlorpromazine (CPZ), trifluoperazine (TFP), and W-7 was measured 7 min after drug introduction via the cannulated vascular system of the VPRH. In separate preparations, %FC was recorded 10 min after the simultaneous administration of the calmodulin inhibitor and the phosphodiesterase inhibitor, theophylline (THEO). *P(t) less than 0.05; **P(t) less than 0.005 as compared to control.

TABLE 2.

EFFECTS OF CALMODULIN ANTAGONISTS ON ACH RELEASE

| TREATMENT | (n) | ACH RELEASE (PM/MIN) |
|---|---|---|
| CONTROL | 9 | 6.52 ± 0.55 |
| FLUPHENAZINE | 4 | 4.19 ± 0.19* |
| FLU + THEO | 6 | 5.85 ± 0.86 |
| CHLORPROMAZINE | 5 | 5.26 ± 0.27* |

Stimulated release of ACh from vascular perfused rat hemidiaphragm as measured by chemiluminescent assay. Drug ($10^{-4}$M) was introduced after 75 min of continuous 10Hz phrenic nerve stimulation established a steady state output of transmitter. Values for release in the presence of drug represent normalized means from three 15 min collection periods. THEO (theophylline) was used at $10^{-4}$M to antagonize fluphenazine. *$P(t) < 0.05$. Values represent mean ± SEM of acetylcholine (ACh) release in pmol/min/hemidiaphragm. n = number of preparations.

Table 3. Specific silver grain densities (total binding - displaced binding) for $^{125}$I- BuTX binding site accumulations at a ligature placed in the rat sciatic nerve for 8 hr. Values are expressed as grains/10 $um^2$.

| TREATMENT | PROXIMAL | | DISTAL | |
|---|---|---|---|---|
| Control | 16.93 | 2.99 | 19.12 | 3.45 |
| Acrylamide | 18.02 | 2.42 | 6.65 | 1.89* |
| IDPN | 6.97 | 0.89* | 7.59 | 0.88* |
| BPAU | 16.48 | 2.82 | 17.69 | 1.66 |
| Crush | 3.23 | | 5.00 | |

* $p<0.05$ Student's T-test.

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